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ORIGINAL ARTICLE

Normal Wound Healing in Mice Deficient for Fibulin-5, an Elastin Binding Protein Essential for Dermal Elastic Fiber Assembly

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Extracellular matrix proteins play a critical role in dermal wound healing by mediating matrix–cell interactions and re-establishing the dermal architecture and environment. Fibulin-5 is an elastin-binding protein essential for elastic fiber development *in vivo*, and it has recently been shown to inhibit angiogenesis *in vitro*. Here, we use mice deficient for the *fibulin-5* gene (*fbln5*) to examine the role of fibulin-5 and the effect of the loss of elastic fibers in dermal wound healing. *Fbln5* is upregulated in the granulation tissue 14 days after full-thickness wounding in wild-type mice, before the formation of elastic fibers. Although wounded *fbln5*$^{-/-}$ skin showed enhanced neovascularization compared to the wild-type skin, no difference in the rate of wound closure was observed between mutant and wild-type mice. In addition, a breaking strength test revealed that there was no difference in breaking stress or strain between wild-type and *fbln5*$^{-/-}$ wounded skin. These results suggest that fibulin-5 and elastic fibers are not directly involved in short-term wound healing. Clearly, the long-term effect of the absence of fibulin-5 on the function and integrity of regenerated skin needs to be further addressed.

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INTRODUCTION

Dermal wound healing is a coordinated process of tissue remodeling involving an inflammatory response, re-epithelialization, and revascularization (reviewed in Singer and Clark 1999). The process is mediated by soluble cytokines and growth factors, which act on multiple cell types including keratinocytes, dermal fibroblasts, and vascular cells. Activated inflammatory cells secrete various matrix proteinases to facilitate breakdown of the extracellular matrix (ECM), which aids in the migration of keratinocytes and fibroblasts into the wound bed (Parks, 1999; Midwood *et al.*, 2004). Deposition of provisional matrices such as fibronectin provides a permissive environment for angiogenesis to occur, which ultimately leads to the healing of the wound and restoration of dermal function.

Collagen and elastic fibers are important components of the dermal ECM and are essential for the maintenance of skin integrity. Skin substitutes containing ECM such as acellular collagen and glycosaminoglycan matrices are routinely used for treatment of acute burns as an alternative to autografting and allografting (Horch *et al.*, 2005). Recent histological analysis, however, has shown that the skin regenerated after using these dressings fails to form adnexal structures and elastic fibers (Moiemen *et al.*, 2001). In another study, epidermal transplantation and dermal matrix substitutes containing both collagen and elastin reduced wound contraction and improved tissue regeneration (Lamme *et al.*, 1996). In order to improve the efficacy of synthetic skin or skin equivalents, the role of each ECM component produced during wound healing needs to be evaluated.

Fibulin-5 is an elastin-binding protein essential for elastic fiber development *in vivo* (Nakamura *et al.*, 2002; Yanagisawa *et al.*, 2002). Various elastogenic cells such as dermal fibroblasts, lung fibroblasts, and vascular smooth muscle cells secrete fibulin-5 and are responsible for tissue-specific elastogenesis. Recently, retrovirus overexpressing *fbln5* was shown to promote wound healing in a full-thickness wound made in rabbit ear by inducing the formation of granulation tissue and remodeling the ECM (Lee *et al.*, 2004). It is not clear, however, whether this effect was owing to an increase in elastic fiber assembly in the wound bed or whether it was mediated through other biological properties of fibulin-5. For instances, fibulin-5 has been shown to antagonize angiogenesis *in vitro* by binding to vascular endothelial growth factor and suppressing downstream signaling (Albig and Schiemann, 2004). Overexpression of *fbln5* can also suppress increased proliferation and migration of *fbln5*$^{-/-}$

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Abbreviations: ECM, extracellular matrix; EGF, epidermal growth factor; fbln5, fibulin-5 gene; FBS, fetal bovine serum

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vascular smooth muscle cells by a yet unknown mechanism (Spencer *et al.*, 2005). Furthermore, fibulin-5 can bind cell surface integrins via an RGD (arginine-glycine-aspartic acid) motif located in the first calcium binding epidermal growth factor (CB-EGF)-like domain (Nakamura *et al.*, 1999). Thus, it is possible that some of the biological effects of fibulin-5 may be related to integrin binding and signaling.

In the present study, we examined whether the absence of fibulin-5 and the presence of abnormal elastic fibers in *fbln5*$^{-/-}$ mouse skin would impact wound closure *in vivo*, or alternatively, whether fibulin-5 plays a key role in dermal wound healing by affecting the properties of dermal cells.

RESULTS

Fibulin-5 is upregulated during wound healing

We first examined the expression of *fbln5* during wound healing in wild-type mice by reverse transcriptase-PCR. RNA was prepared from the dorsal skin before and after full-thickness excision wounding. As shown in Figure 1a, adult skin expressed a low level of *fbln5* and the expression was significantly upregulated at day 14. Immunostaining for fibulin-5 showed that the upper dermis, perifollicular region, and the blood vessel walls of small arteries (data not shown) were positive for fibulin-5 in non-wounded skin (Figure 1b). Fourteen days after wounding, granulation tissue forming the wound bed, including newly formed vessels, was intensely stained with fibulin-5 (Figure 1d and f). The regenerative hyperplastic epidermis was also positive for fibulin-5 (Figure 1f). On adjacent sections, elastin immunoreactivity was unchanged (data not shown). Control sections from *fbln5*$^{-/-}$ mice showed no staining for fibulin-5, although the part of epidermis gave a false-positive reaction (Figure 1c, e, and g). These data indicated that fibulin-5 is upregulated in the granulation tissues during the subacute phase of wound healing.

Wound closure was not affected in *fbln5*$^{-/-}$ mice

In previous work, we observed an exacerbated vascular injury response in *fbln5*$^{-/-}$ mice following flow cessation-induced injury to the carotid artery (Spencer *et al.*, 2005). We therefore wanted to determine whether the compromised elastic fibers in *fbln5*$^{-/-}$ mice would affect wound healing *in vivo*, and whether the absence of fibulin-5 would increase migration and proliferation of vascular cells and fibroblasts into the wound bed. *Fbln5*$^{-/-}$ and wild-type littermates were subjected to full-thickness excision wounding in the dorsal skin and wound healing was photographed on the days indicated (Figure 2a). At six different time points, the area of the wound was measured and expressed as a percentage of the initial wound size (Figure 2b). No statistical difference was found at any time point between wild-type and *fbln5*$^{-/-}$ mice and wounds from both genotypes were closed by day 14. Macroscopically, we observed excessive skin folds running in multiple directions in *fbln5*$^{-/-}$ mice compared to wild-type mice (see Figure 2a, day 14). Upon histological examination, both wild-type and *fbln5*$^{-/-}$ skin showed re-epithelialization and dermal fibrosis (Figure 2c and f). No difference was detected in the distribution or intensity of collagen fibers (Figure 2d and g). Elastin staining revealed that

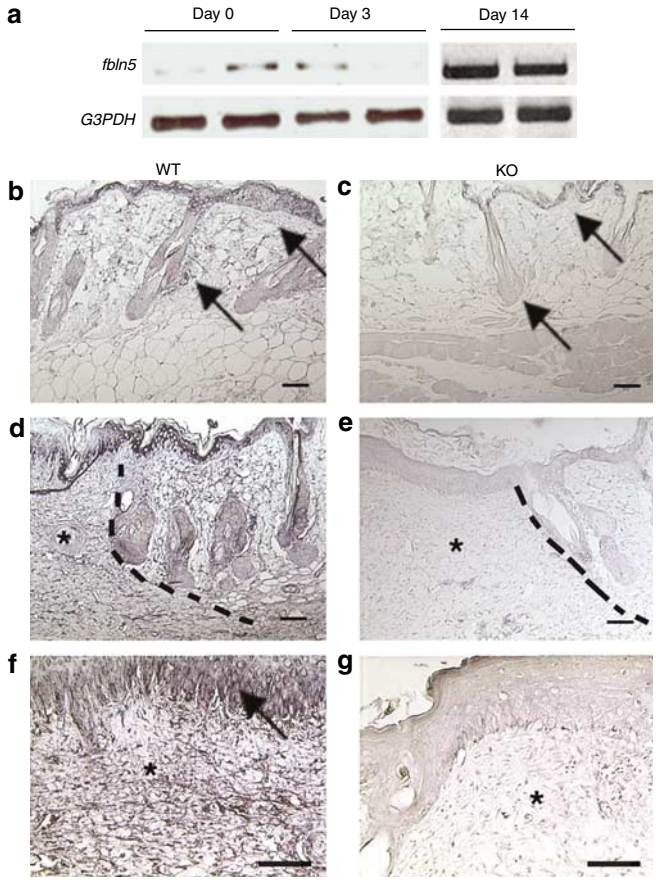

Figure 1. Fibulin-5 expression and localization during skin excision wound healing. (**a**) Upregulation of *fbln5* during dermal wound healing. Reverse transcriptase-polymerase chain reaction was performed using RNA prepared from the dorsal skin harvested before (day 0) and after (days 3 and 14) full-thickness excision wounding in wild-type mice. Glycerol-3-phosphate dehydrogenase was used as internal control. There was no consistent increase in *fbln5* expression at early phase of wound healing, whereas the significant upregulation was observed at 14 days. (**b–g**) Detection of fibulin-5 by immunohistochemistry. Sections of adult dorsal skin were stained with anti-fibulin-5 antibody before (**b**, **c**) or 14 days post-wounding (**d–g**) in wild-type (WT; **b**, **d**, **f**) and *fbln5*$^{-/-}$ (KO; **c**, **e**, **g**) mice. Fibulin-5 was expressed around hair follicles, and to a lesser extent in the upper dermis before wounding (**b**, arrows). After wounding, robust fibulin-5 immunoreactivity was detected in the granulation tissue in the wild-type mouse (**d**, **f**, asterisks). Similar areas were not stained in *fbln5*$^{-/-}$ skin (**c**, arrows; **e**, **g**, asterisks). Regenerated epidermis (**f**, arrow) expressed fibulin-5. Dotted line separates wounded and surrounding dermis. Bar = 200 μm.

elastic fibers had not yet regenerated in the wound bed of wild-type skin, whereas dense elastic fibers were observed in non-injured dermis adjacent to the wound (Figure 2e, arrows). In *fbln5*$^{-/-}$ mice, no filamentous network of elastic fibers was seen in the dermis within or outside of wound bed (Figure 2h). These data indicate that absence of fibulin-5 does not positively or negatively affect wound closure *in vivo*.

Fibulin-5 does not affect migration or proliferation of dermal cells *in vitro*

In order to examine if fibulin-5 affects dermal cell function, primary skin fibroblasts from newborn wild-type and *fbln5*$^{-/-}$

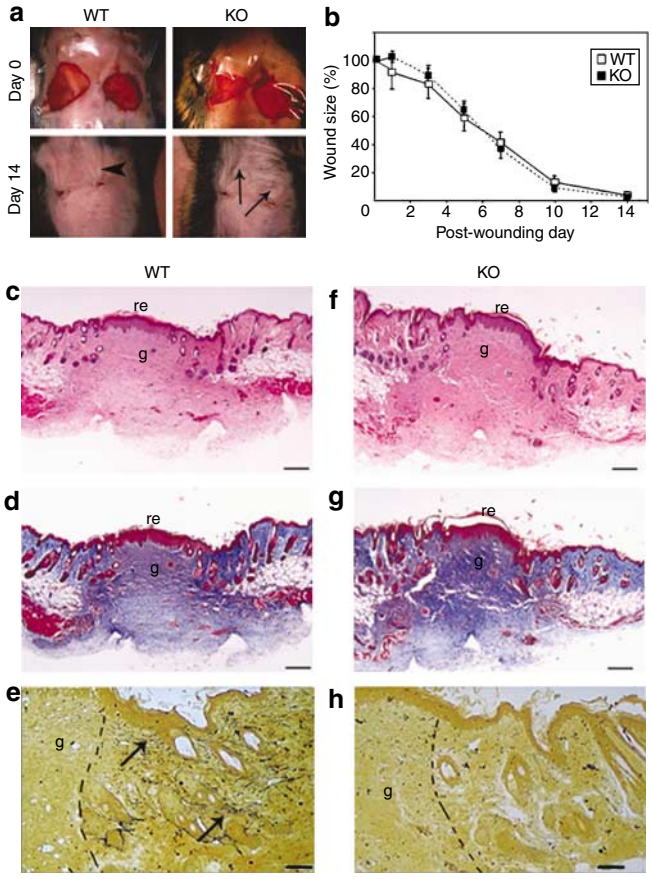

Figure 2. Macroscopic and histological observations of wound closure. (**a**) Representative photographs showing the wounds in wild-type (WT) and *fbln5*$^{-/-}$ mice at indicated time after wounding. Note increased skin folds in a random direction in the *fbln5*$^{-/-}$ mouse (arrows) at day 14 compared to the wild-type mouse (arrowhead). (**b**) Quantification of wound closure. Wound areas were measured on days 1, 3, 5, 7, 10, and 14 after wounding and expressed as percentage of the initial wound size (day 0). No statistically significant difference was observed at any time point ($P > 0.05$). Open squares indicate the wild-type ($n = 7$) and closed squares indicate *fbln5*$^{-/-}$ mice ($n = 7$). Bars indicate means $\pm$ SD. (**c**–**h**) Histological examination of the wild-type and *fbln5*$^{-/-}$ skin 14 days after wounding. (**c**, **f**) Hematoxylin and eosin staining shows granulation tissues (g) devoid of hair follicles and overlying regenerated epidermis (re). No difference is evident between wild-type (**c**) and *fbln5*$^{-/-}$ mice (**f**). (**d**, **g**) Masson-Trichrome staining for collagen fibers appears similar between the two genotypes. (**e**, **h**) Hart's staining for elastic fibers shows a network of elastic fibers in the dermis adjacent to the wound in the wild-type skin (**e**, arrows), which is not seen in the *fbln5*$^{-/-}$ skin (**h**). No elastic fibers are formed in the granulation tissue in either animal (g). Dotted lines indicate the border between the wound and adjacent dermis. Bar = 400 μm (**c**, **d**, **f**, **g**) and 200 μm (**e**, **h**).

pups were isolated and the effect of fibulin-5 on proliferation and migration was assessed. After synchronization, cells were stimulated with EGF (10 ng/ml) for 24 hours and cell number was assessed by colormetric assay. No difference in proliferative response to EGF between wild-type and *fbln5*$^{-/-}$ cells was seen (Figure 3a). Scratch assays performed in the presence of EGF showed a modest increase in migratory response in wild-type and *fbln5*$^{-/-}$ cells over control cultures. Stimulation with 20% fetal bovine serum (FBS) induced a strong migratory response; however, no significant difference was observed between cells from the two genotypes (Figure 3b). Western analysis demonstrated similar activation of downstream signaling pathways involving phosphorylated extracellular signal-regulated kinase1/2, c-Jun N-terminal kinase, and focal adhesion kinase (Figure 3c), and stimulation with platelet-derived growth factor (20 ng/ml) showed the same results as with serum (data not shown). Similar results were obtained using mouse embryonic fibroblasts (data not shown).

We next examined the potential of skin keratinocytes to epithelialize using a skin *ex vivo* assay. Outgrowth of keratinocytes from the skin explants was evaluated after 5 days and keratinocytes were stained with an anticytokeratin antibody (Figure 3d). Total migration area showed no difference between wild-type and *fbln5*$^{-/-}$ explants, consistent with our *in vivo* data (Figure 3e).

Dermal tensile strength

To determine if the biomechanical function of the wound bed was maintained in *fbln5*$^{-/-}$ mice, breaking strength testing was performed using dorsal skin harvested 14 days after incision wounding. No difference was observed in incisional wound closure between wild-type and *fbln5*$^{-/-}$ mice (data not shown). There was also no significant difference in skin thickness between wild-type and *fbln5*$^{-/-}$ mice; however, the skin from the wounds was significantly thicker than the contralateral unwounded control skin in both groups (compare Figure 4c and f). The skin was subjected to uniaxial extension, in which the force was applied perpendicular to the wound. The average breaking stress in unwounded wild-type skin was lower than *fbln5*$^{-/-}$ skin; however, this was not statistically significant ($P = 0.55$, unpaired *t*-test; Figure 4a). The mean strain at breaking was also similar (Figure 4b). The breaking stress was reduced by an order of magnitude upon wounding in both the wild-type and *fbln5*$^{-/-}$ mice (Figure 4d). The breaking strain was similarly reduced, but not significantly different for both wounded wild-type and *fbln5*$^{-/-}$ mouse skin (Figure 4d and e). These data indicated that the breaking strength of wounded skin was not affected by the absence of fibulin-5.

Increased angiogenesis did not facilitate wound closure in *fbln5*$^{-/-}$ mice

Neovascularization is an important component of wound healing, and conditions with impaired angiogenesis, such as diabetes, are known to cause a delay in healing (Falanga, 2005). In previously reported work, we established that fibulin-5 is an inhibitory matrix to vascular smooth muscle cells (Spencer *et al.*, 2005). It was of interest, therefore, to determine if the absence of fibulin-5 would result in an accelerated or increased vascular response in the wound bed. We first performed CD31 immunostaining on sections of non-injured skin from wild-type and *fbln5*$^{-/-}$ mice. As seen in Figure 5(a–d), an increased number of CD31-positive dermal vessels were observed in *fbln5*$^{-/-}$ skin, with vessels that were wider and more tortuous compared to those in wild-type dermis. The same result was obtained using α-smooth muscle actin immunostaining (data not shown), indicating that there

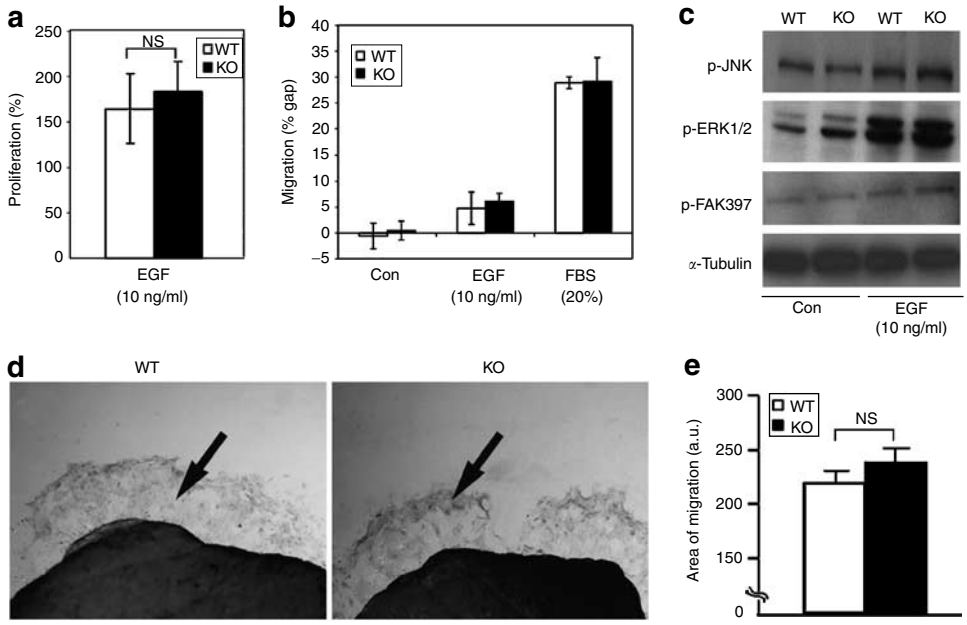

Figure 3. Effects of fibulin-5 on proliferation and migration of dermal cells. (**a**) Proliferation assays using wild-type and *fbln5*$^{-/-}$ skin fibroblasts measured after 24 hours of serum starvation followed by 24 hours of stimulation with EGF (10 ng/ml). Data are indicated as percentage relative to cell numbers measured without stimulation. Bars indicate means ± SD. NS, not significant. (**b**) Scratch assays using wild-type and *fbln5*$^{-/-}$ skin fibroblasts performed in serum-free medium (control), or with EGF (10 ng/ml) or 20% FBS. No difference between the two genotypes was seen. (**c**) Intracellular signaling induced by EGF (10 ng/ml) in wild-type and *fbln5*$^{-/-}$ skin fibroblasts. Phospho-c-Jun N-terminal kinase and extracellular signal-regulated kinase1/2 were significantly increased in both wild-type and *fbln5*$^{-/-}$ cells. Phospho-FAK was increased in a lesser degree. No significant difference was seen between the genotypes. (**d**) Representative photos of skin explants from wild-type ($n = 4$) and *fbln5*$^{-/-}$ ($n = 3$) mice. Keratinocytes were stained with anti-cytokeratin (arrows). (**e**) Area of migrating keratinocytes was measured using ImageJ software.

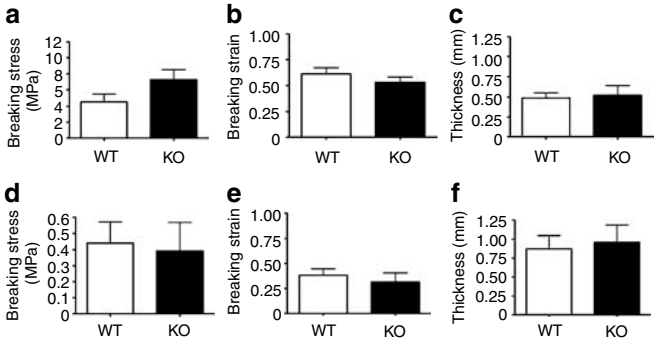

Figure 4. Breaking strength of full-thickness incision wounds. Fourteen days following full-thickness incision wounding, the wounded skin (**d–f**) and uninjured contralateral skin (**a–c**) were harvested and subjected to breaking testing. Breaking stress (**a**, **d**) and strain (**b**, **e**) at the breaking point were measured. (**c**, **f**) Average skin thickness. Wild-type ($n = 6$) and *fbln5*$^{-/-}$ mice ($n = 8$) were examined. All graphs show means ± SEM.

was increased angiogenesis owing to the absence of fibulin-5 during embryonic and/or postnatal development. This result is consistent with our macroscopic observation of an increased number of vascular branches sprouting from the long thoracic artery in the skin of *fbln5*$^{-/-}$ mice compared to wild-type mice (Sullivan *et al.*, manuscript in preparation). Fourteen days after injury, increased angiogenesis was observed in both wild-type and *fbln5*$^{-/-}$ dermis compared to non-injured skin (Figure 5a–d). The total number of vessels formed in response to wounding, however, was significantly higher in *fbln5*$^{-/-}$ skin compared to the wild-type skin (Figure 5e). Interestingly, we did not detect any difference in the expression level of vascular endothelial growth factor isoforms, angiopoietin 1 and 2, or cardiac ankyrin repeat protein, which has been implicated in the induction of angiogenesis (data not shown) (Shi *et al.*, 2005) in the wounded bed. Overall, these results suggest that a fibulin5-deficient microenvironment may be more permissive to angiogenesis.

DISCUSSION

Fbln5 knockout mice were used to address the role of fibulin-5 and elastic fibers in dermal wound healing. We also tested whether a loss of fibulin-5 attenuates or exacerbates the dermal injury response by acting on dermal cells and/or vascular cells. Our present data showed that the absence of fibulin-5 and an underlying elastic fiber defect does not affect short-term wound closure or tensile strength of the wounded skin. These results indicate that fibulin-5 does not play a critical role in the wound healing process.

Dermal elastic fibers and wound healing

Migration and proliferation of keratinocytes and fibroblasts, and ECM-induced contraction of fibroblasts contribute to wound closure. We observed that elastic fibers were not

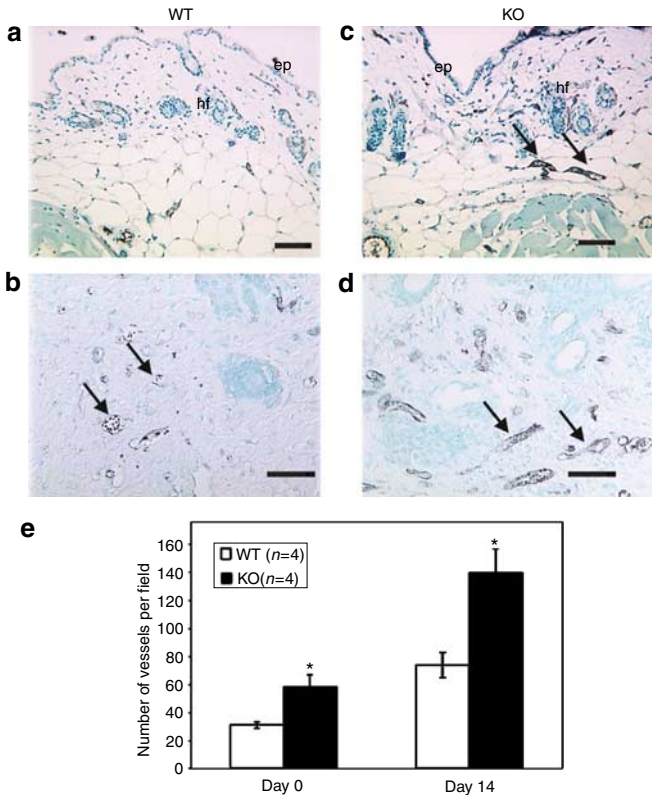

Figure 5. Increased blood vessel formation during wound healing in *fbln5*$^{-/-}$ mice. (**a**–**d**) CD31 immunostaining in the skin of wild-type (**a**, **b**) and *fbln5*$^{-/-}$ (**c**, **d**) mice before (**a**, **c**) and 14 days post-wounding (**b**, **d**). Sections are counterstained with methyl green. Tortuous vessels are observed in the *fbln5*$^{-/-}$ skin even before wounding (**c**, arrows). The number of vessels increased in the *fbln5*$^{-/-}$ skin (**d**, arrows) compared to wild type (**b**, arrows) after wounding. Bar = 200 μm. (**e**) Quantification of dermal vessels in wild-type and *fbln5*$^{-/-}$ skin before and 14 days post-wounding. A minimum of four images of non-injured dermis and granulation tissues were captured from each section and vessels were counted. Four sections per animal were quantified from four animals per genotype. Bars indicate means ± SD. *$P < 0.05$.

detected in the granulation tissue at 14 days post-wounding in wild-type mice, and the comparable speed of wound closure between wild-type and *fbln5*$^{-/-}$ mice indicated that elastic fibers were not required for early phases of wound healing. Our results also demonstrated that the absence of elastic fibers in neighboring tissues did not affect migration of keratinocytes or dermal fibroblasts into the wound bed, nor affected the formation of granulation tissue.

Elastic fibers play an important role in maintenance of the skin integrity. The fibers are organized in an elaborate three-dimensional network in the dermis where they provide elasticity and recoil. In an early study, granulation tissue was found to be devoid of elastin staining and thus elastic fibers were thought not to be actively involved in the wound healing process (Montagna *et al.*, 1988). Another study, however, showed that elastin and fibrillin-1 were deposited in the deep wound bed 7 days post-injury and expression persisted for 21 days (Ashcroft *et al.*, 1997). In a keratinocyte autograft model, elastin deposition was shown to appear first at the dermo-epidermal junction preceding elastic fiber formation (Raghunath *et al.*, 1996). Overall, our *in vivo* findings together with previous reports confirmed that elastic fibers do not participate in the wound closure process itself, rather they may play a supportive role in re-establishment of dermal architecture.

Role of fibulin-5 during wound healing

Although production and deposition of fibulin-5 was significantly upregulated in the granulation tissue before the formation of elastic fibers, we did not detect any differences in breaking strength between the wild-type and *fbln5*$^{-/-}$ skin. Breaking stress and strain are affected by various factors, most notably by collagen fibers. Inactivation of genes involved in collagen assembly or maintenance, including decorin, dermatopontin, and thrombospondin 2, is known to exhibit reduced tensile strength (Danielson *et al.*, 1997; Kyriakides *et al.*, 1998; Takeda *et al.*, 2002). Our observation that collagen fibers are not morphologically affected by the absence of fibulin-5 (J. Choi *et al.*, unpublished observation), together with our previous finding that fibulin-5 did not bind type I collagen *in vitro* (Yanagisawa *et al.*, 2002), suggests that fibulin-5 is not involved in collagen assembly or collagen–matrix interactions.

In a previous study, retrovirus-mediated gene transduction of *fbln5* in a full-thickness wound was reported to simulate granulation formation and facilitated wound closure in rabbit ear (Lee *et al.*, 2004). Increased production of type I collagen by fibulin-5 was suggested to be a part of the mechanism for the accelerated wound closure. In contrast, our loss-of-function study does not support this notion; however, it is plausible that a net change in the amount of ECM may have affected the outcome of ECM-mediated cell signaling.

Angiogenesis and fibulin-5

Fbln5$^{-/-}$ skin showed an enhanced neovascularization before and during wound healing compared to wild-type skin. As we have not observed increased vessel formation or altered vascular patterning during embryonic development (H Yanagisawa, unpublished observation), the absence of fibulin-5 may just result in a more permissive environment for angiogenesis during postnatal development. Consistent with this notion, we see a general increase in small cutaneous vessels in *fbln5*$^{-/-}$ skin in older animals (Sullivan *et al.*, manuscript in preparation).

It has recently been shown that sprouting endothelial cells and pericytes use elastic fibers as a scaffold for migration in rat mesenteric connective tissues (Anderson *et al.*, 2004). Although the dependence on elastic fibers as tracks for sprouting is reduced during physiological wound healing or after chemical induced angiogenesis, over 60% of sprouts are still in direct contact with elastic fibers during these processes. Thus, it is possible that fibulin-5-containing fibers may also serve as a track for invading small vessels. Consistent with this notion, fibulin-5 has been shown to form fibers by binding to pre-existing matrices (Zheng *et al.*, manuscript in preparation), and the binding partners are not limited to elastin (Freeman *et al.*, 2005).

Of interest is the fact that increased neovascularization in *fbln5*$^{-/-}$ mice did not facilitate wound healing. A strong relationship between angiogenesis and wound healing has been reported for mice deficient for endothelial nitric oxide synthase, syndecan-4, and thrombospondin 2 (Kyriakides *et al.*, 1999; Lee *et al.*, 1999; Echtermeyer *et al.*, 2001). In addition, therapeutic strategies to facilitate angiogenesis during wound healing have been considered using thymosin 4, vascular endothelial growth factor, and FGF ligands (Malinda *et al.*, 1999; Simons, 2005). Our current study offers an interesting example where increased angiogenesis does not promote more rapid wound healing. However, vascularization may be critical for more complex wound healing processes such as with an underlying diabetic condition (Galiano *et al.*, 2004). Further analysis of the direct role of fibulin-5 on endothelial function will be necessary to establish a molecular basis of the role of fibulin-5 in angiogenesis.

MATERIALS AND METHODS

Mice

Fbln5$^{-/-}$ mice were maintained on a C57BL6 × 129/SvEv hybrid background and kept under a 12-hours/12-hours light cycle in specific pathogen-free condition (Yanagisawa *et al.*, 2002). Wild-type or heterozygous littermates were used as controls. All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committees at the University of Texas Southwestern Medical Center.

Reverse transcriptase-polymerase chain reaction

Total RNA was isolated from dorsal skin before and after wounding using Trizol reagent (Invitrogen, Grand Island, NY) according to the manufacturer's protocol. RNA was treated with DNase I (RNase free, Ambion, Austin, TX), followed by first-strand synthesis using randam hexamer primers (Bio-Rad, Hercules, CA) from 1 μg of total RNA. PCR was performed with 2 μl of the cDNA for 30 cycles (30 seconds at 94°C, 30 seconds at 55°C, and 30 seconds at 72°C) for amplification of *fbln5* and *G3PDH*. Sequences of primers are available upon request.

Histology

Hematoxylin and eosin staining was used for routine histological observations. Modified Hart's and Masson-Trichrome staining were used for visualization of elastic fibers and collagen fibers, respectively.

Skin explants culture

The medium was prepared as described previously (Mazzalupo *et al.*, 2002) with modifications. DMEM was mixed with Ham's F-12 medium (Invitrogen) in a 3:1 ratio and supplemented with EGF (10 ng/ml; Roche, Germany), cholera toxin (0.1 nM), T3 (2 nM), transferrin (5 μg/ml), insulin (5 μg/ml), hydrocortisone (0.4 μg/ml) (all from Sigma, St Louis, MO), penicillin (100 U/ml), streptomycin (100 μg/ml) (Invitrogen), and 10% FBS (Gemini, Woodland, CA). Postnatal day (P) 2 wild-type and *fbln5*$^{-/-}$ pups were killed and the whole dorsal skin was harvested. Two 8 mm sterile punches were made from each pup and laid flat individually into a 12-well tissue culture dish, dermis side down. Explants were incubated at 37°C for 5 minutes to let skin adhere. Four hundred microliters of medium was added into each well. The explants were submerged by adding 1.5 ml/well medium on the following day. Keratinocytes were stained with anti-cytokeratin (Biolegend, San Diego, CA) at 1:50 dilution, followed by biotinylated goat anti-mouse 1:200 and diaminobenzidine substrate. Total area of migrated keratinocytes was measured and quantified by image J image analysis software (NIH).

Immunohistochemistry

Skin tissue was immersion fixed in 4% paraformaldehyde overnight at 4°C for fibulin-5 and elastin staining, and in zinc fixative solution (Beckstead, 1994) for 24 hours at room temperature for CD31 and α-smooth muscle actin staining. Sections (10 μm) were deparaffinized and rehydrated in alcohol gradients. After rinsing with phosphate-buffered saline, sections were treated with 1% H_2O_2 for 10 minutes at room temperature and blocked in 3% BSA and 1% normal goat serum. Primary antibodies for fibulin-5, raised against recombinant full-length rat fibulin-5, and for elastin (provided by Dr Robert Mecham, Washington University School of Medicine) were diluted 1:200, anti-CD31 (BD Biosciences Pharmingen, San Diego, CA) was diluted 1:50, and anti-α-smooth muscle actin (Sigma) was diluted 1:500 in blocking solution and incubated at 4°C overnight. Slides were washed 3 × 10 minutes with phosphate-buffered saline and incubated with biotinylated anti-rabbit (fibulin-5 and elastin), anti-rat (CD31), or anti-mouse (α-smooth muscle actin) secondary antibodies (1:200; Vector Labs, Burlingame, CA) for 1 hour at room temperature. Slides were washed as described above. Streptavidin-conjugated horseradish peroxidase and diaminobenzidine substrate were used as directed by the manufacturer (ABC and DAB Kit, Vector Labs, Burlingame, CA).

Cells

Primary skin fibroblasts were prepared from the dorsal skin of P1 wild-type and *fbln5*$^{-/-}$ pups as described previously (Yanagisawa *et al.*, 2002). Cells were cultured in DMEM containing 10% FBS, streptomycin (100 μg/ml), and penicillin-G (100 U/ml).

In vitro proliferation assays

Wild-type and *fbln5* $^{-/-}$ skin fibroblasts were plated in 96-well dishes (2,000 cells/well) and serum-starved for 24 hours to synchronize the cell cycle and then stimulated with EGF (10 ng/ml). Cell numbers were quantified using Cell Titer 96 Aqueous One Solution (Promega, Madison, WI) according to the manufacturer's instructions. Three independent experiments were performed in duplicate and the results were presented as the fold-increase over non-stimulated wild-type cells at 24 hours to allow for comparison between experiments.

In vitro scratch assays

Wild-type and *fbln5* $^{-/-}$ skin fibroblasts were plated at approximately 80% confluency in six-well dishes. Cells were allowed to reach confluence and serum-starved for 24 hours. A sterile 1 ml pipette tip was used to administer the scratch injury. Media were changed to DMEM containing no additives, 10 ng/ml EGF, or 20% FBS. Cells were allowed to migrate into the cleared zone for 24 hours and fixed in 4% paraformaldehyde. Images were captured and cells were scored based on the distance migrated from the border of the

scratch injury. Two independent experiments were performed in duplicate for skin fibroblasts. Four to five separate images were scored for each condition.

In vivo wound healing assays

Excision wound healing experiments were performed as previously described with some modifications (Crowe *et al*., 2000). Briefly, 8–12-week-old wild-type and *fbln5*$^{-/-}$ mice were anesthesized and two full-thickness skin punch biopsies of 0.8 cm were created using a disposable dermal biopsy punch (Miltex, York, PA). Wounds were covered with a semipermeable polyurethane nonabsorbent dressing (OpSite, Smith & Nephew, Largo, FL) and sealed at the edge. Skin wounds were documented on postoperative day 0, 1, 3, 5, 7, 10, and 14 using a digital camera and the wound area was measured using Image J software (NIH).

Tensile strength

Wild-type and *fbln5*$^{-/-}$ male mice (12- to 20-week-old) were anesthetized and one full-thickness incision (2.0 cm) was created parallel to the spinal column and the wound was staple-closed and allowed to heal for 14 days. Dorsal skin from the wounded and contralateral sides were harvested and trimmed into an ASTM standard dog-bone shape for tensile testing. The thickness of the samples was taken at four random locations using a constant force thickness indicator (Mitutoyo, Litematic VL-50A, $\pm$0.0 001 mm) and recorded. The sample dimensions were verified using a handheld digital micrometer (Mitutoyo, Series 500, $\pm$0.01 mm). The breaking stress and breaking strain were determined from the engineering stress–strain curve acquired with an EnduraTEC ELectroForce® 3200 soft tissue tester (Bose Corporation, Eden Prairie, Minnesota). Briefly, samples were fastened between two clamps with 12 mm gauge length (the distance between the clamps) and longitudinally stretched along their length under displacement control at a constant strain rate of 0.1 mm/second. Time, force, and displacement were recorded for stretching to failure.

Statistical analysis

Statistical analysis between genotypes was performed by unpaired Student's *t*-test. *P*-value less than 0.05 was considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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REFERENCES

Albig AR, Schiemann WP (2004) Fibulin-5 antagonizes vascular endothelial growth factor (VEGF) signaling and angiogenic sprouting by endothelial cells. *DNA Cell Biol* 23:367–79

Anderson CR, Ponce AM, Price RJ (2004) Immunohistochemical identification of an extracellular matrix scaffold that microguides capillary sprouting *in vivo*. *J Histochem Cytochem* 52:1063–72

Ashcroft GS, Horan MA, Herrick SE, Tarnuzzer RW, Schultz GS, Ferguson MW (1997) Age-related differences in the temporal and spatial regulation of matrix metalloproteinases (MMPs) in normal skin and acute cutaneous wounds of healthy humans. *Cell Tissue Res* 290:581–91

Beckstead JH (1994) A simple technique for preservation of fixation-sensitive antigens in paraffin-embedded tissues. *J Histochem Cytochem* 42:1127–34

Crowe MJ, Doetschman T, Greenhalgh DG (2000) Delayed wound healing in immunodeficient TGF-beta 1 knockout mice. *J Invest Dermatol* 115:3–11

Danielson KG, Baribault H, Holmes DF, Graham H, Kadler KE, Iozzo RV (1997) Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility. *J Cell Biol* 136:729–43

Echtermeyer F, Streit M, Wilcox-Adelman S, Saoncella S, Denhez F, Detmar M *et al.* (2001) Delayed wound repair and impaired angiogenesis in mice lacking syndecan-4. *J Clin Invest* 107:R9–14

Falanga V (2005) Wound healing and its impairment in the diabetic foot. *Lancet* 366:1736–43

Freeman LJ, Lomas A, Hodson N, Sherratt MJ, Mellody KT, Weiss AS *et al.* (2005) Fibulin-5 interacts with fibrillin-1 molecules and microfibrils. *Biochem J* 388(Part 1):1–5

Galiano RD, Tepper OM, Pelo CR, Bhatt KA, Callaghan M, Bastidas N *et al.* (2004) Topical vascular endothelial growth factor accelerates diabetic wound healing through increased angiogenesis and by mobilizing and recruiting bone marrow-derived cells. *Am J Pathol* 164:1935–47

Horch RE, Kopp J, Kneser U, Beier J, Bach AD (2005) Tissue engineering of cultured skin substitutes. *J Cell Mol Med* 9:592–608

Kyriakides TR, Tam JW, Bornstein P (1999) Accelerated wound healing in mice with a disruption of the thrombospondin 2 gene. *J Invest Dermatol* 113:782–7

Kyriakides TR, Zhu YH, Smith LT, Bain SD, Yang Z, Lin MT *et al.* (1998) Mice that lack thrombospondin 2 display connective tissue abnormalities that are associated with disordered collagen fibrillogenesis, an increased vascular density, and a bleeding diathesis. *J Cell Biol* 140:419–30

Lamme EN, de Vries HJ, van Veen H, Gabbiani G, Westerhof W, Middelkoop E (1996) Extracellular matrix characterization during healing of full-thickness wounds treated with a collagen/elastin dermal substitute shows improved skin regeneration in pigs. *J Histochem Cytochem* 44:1311–22

Lee MJ, Roy NK, Mogford JE, Schiemann WP, Mustoe TA (2004) Fibulin-5 promotes wound healing *in vivo*. *J Am Coll Surg* 199:403–10

Lee PC, Salyapongse AN, Bragdon GA, Shears LL II, Watkins SC, Edington HD *et al.* (1999) Impaired wound healing and angiogenesis in eNOS-deficient mice. *Am J Physiol* 277(4 Part 2):H1600–8

Malinda KM, Sidhu GS, Mani H, Banaudha K, Maheshwari RK, Goldstein AL *et al.* (1999) Thymosin beta4 accelerates wound healing. *J Invest Dermatol* 113:364–8

Mazzalupo S, Wawersik MJ, Coulombe PA (2002) An *ex vivo* assay to assess the potential of skin keratinocytes for wound epithelialization. *J Invest Dermatol* 118:866–70

Midwood KS, Williams LV, Schwarzbauer JE (2004) Tissue repair and the dynamics of the extracellular matrix. *Int J Biochem Cell Biol* 36:1031–7

Moiemen NS, Staiano JJ, Ojeh NO, Thway Y, Frame JD (2001) Reconstructive surgery with a dermal regeneration template: clinical and histologic study. *Plast Reconstr Surg* 108:93–103

Montagna W, Carlisle K, Brenner RM (1988) Wound healing in the sex skin of pig-tailed macaques. *Arch Dermatol Res* 280(Suppl):S68–84

Nakamura T, Lozano PR, Ikeda Y, Iwanaga Y, Hinek A, Minamisawa S *et al.* (2002) Fibulin-5/DANCE is essential for elastogenesis *in vivo*. *Nature* 415:171–5

Nakamura T, Ruiz-Lozano P, Lindner V, Yabe D, Taniwaki M, Furukawa Y *et al.* (1999) DANCE, a novel secreted RGD protein expressed in developing, atherosclerotic, and balloon-injured arteries. *J Biol Chem* 274:22476–83

Parks WC (1999) Matrix metalloproteinases in repair. *Wound Repair Regen* 7:423–32

Raghunath M, Bachi T, Meuli M, Altermatt S, Gobet R, Bruckner-Tuderman L *et al.* (1996) Fibrillin and elastin expression in skin regenerating from

cultured keratinocyte autografts: morphogenesis of microfibrils begins at the dermo-epidermal junction and precedes elastic fiber formation. *J Invest Dermatol* 106:1090–5

Shi Y, Reitmaier B, Regenbogen J, Slowy R, Opalenik S, Wolf E *et al.* (2005) CARP, a cardiac ankyrin repeat protein, is up-regulated during wound healing and induces angiogenesis in experimental granulation tissue. *Am J Pathol* 166:303–12

Simons M (2005) Angiogenesis: where do we stand now? *Circulation* 111:1556–66

Singer AJ, Clark RA (1999) Cutaneous wound healing. *N Engl J Med* 341:738–46

Spencer JA, Hacker SL, Davis EC, Mecham RP, Knutsen RH, Li DY *et al.* (2005) Altered vascular remodeling in fibulin-5-deficient mice reveals a role of fibulin-5 in smooth muscle cell proliferation and migration. *Proc Natl Acad Sci USA* 102:2946–51

Takeda U, Utani A, Wu J, Adachi E, Koseki H, Taniguchi M *et al.* (2002) Targeted disruption of dermatopontin causes abnormal collagen fibrillogenesis. *J Invest Dermatol* 119:678–83

Yanagisawa H, Davis EC, Starcher BC, Ouchi T, Yanagisawa M, Richardson JA *et al.* (2002) Fibulin-5 is an elastin-binding protein essential for elastic fibre development *in vivo*. *Nature* 415:168–71